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CYCLIC NUCLEOTIDE PHOSPHODIESTERASES

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DNA ENCODING MAMMALIAN PHOSPHODIESTERASES

This is a continuation-in-part of our co-pending U.S. Patent Application Serial No. 07/688,356, filed April 19, 1991.

BACKGROUND OF THE INVENTION

The present invention relates to novel purified and isolated nucleotide sequences encoding mammalian Ca^{2+}/calmodulin stimulated phosphodiesterases (CaM-PDEs) and cyclic-GMP-stimulated phosphodiesterases (cGS-PDEs). Also provided are the corresponding recombinant expression products of said nucleotide sequences, immunological reagents specifically reactive therewith, and procedures for identifying compounds which modulate the enzymatic activity of such expression products.

Cyclic nucleotides are known to mediate a wide variety of cellular responses to biological stimuli. The cyclic nucleotide phosphodiesterases (PDEs) catalyze the hydrolysis of 3′, 5′ cyclic nucleotides, such as cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), to their corresponding 5′-nucleotide monophosphates and are consequently important in the control of cellular concentration of cyclic nucleotides. The PDEs in turn are regulated by transmembrane signals or second messenger ligands such as calcium ion (Ca^{2+}) or cGMP. The PDEs thus have a central role in regulating the flow of information from extracellular hormones, neurotransmitters, or other signals that use the cyclic nucleotides as messengers.

PDEs are a large and complex group of enzymes. They are widely distributed throughout the cells and tissues of most eukaryotic organisms, but are usually present only in trace amounts. At least five different

families of PDEs have been described based on characteristics such as substrate specificity, kinetic properties, cellular regulatory control, size, and in some instances, modulation by selective inhibitors. [Beavo, Adv. in Second Mess. and Prot. Phosph. Res. 22:1-38 (1988)]. The five families include:

I	Ca^{2+}/calmodulin-stimulated
II	cGMP-stimulated
III	cGMP-inhibited
IV	cAMP-specific
V	cGMP-specific

Within each family there are multiple forms of closely related PDEs. See Beavo, "Multiple Phosphodiesterase Isozymes Background, Nomenclature and Implications", pp. 3-15; Wang et al., "Calmodulin-Stimulated Cyclic Nucleotide Phosphodiesterases", pp. 19-59; and Manganiello et al., "Cyclic GMP-Stimulated Cyclic Nucleotide Phosphodiesterases" pp. 62-85; all in Cyclic Nucleotide Phosphodiesterases: Structure, Regulation and Drug Action, Beavo, J. and Houslay, M.D., Eds.; John Wiley & Sons, New York (1990).

The Ca^{2+}/calmodulin dependent PDEs (CaM-PDEs) are characterized by their responsiveness to intracellular calcium, which leads to a decreased intracellular concentration of cAMP and/or cGMP. A distinctive feature of cGMP-stimulated phosphodiesterases (cGS-PDEs) is their capacity to be stimulated by cGMP in effecting cAMP hydrolysis.

In vitro studies have shown increased PDE activity in response to Ca^{2+}/calmodulin in nearly every mammalian tissue studied, as well as in Drosophila, Dictyostelium, and trypanosomes. The level of CaM-PDE in tissues and cellular and subcellular compartments varies

widely. Most cells contain at least a small amount of CaM-PDE activity, with the highest tissue levels being found in the brain, particularly in the synaptic areas. Greenberg et al. Neuropharmacol., 17:737-745 (1978) and Kincaid et al., PNAS (USA), 84:1118-1122 (1987). A decrease in cAMP in astrocytoma cells in response to muscarinic stimulation may be due to calcium dependent increases in CaM-PDE activity. Tanner et al., Mol. Pharmacol., 29:455-460 (1986). Also, CaM-PDE may be an important regulator of cAMP in thyroid tissue. Erneux et al., Mol. Cell. Endocrinol., 43:123-134(1985).

Early studies suggested that there are distinct tissue-specific isozymes of CaM-PDEs. Several members of the CaM-PDE family have now been described, including a 59 kDa isozyme isolated from bovine heart, and 61 and 63 kDa isozymes isolated from bovine brain. LaPorte et al., Biochemistry, 18:2820-2825 (1979); Hansen et al., Proc. Natl. Acad. Sci. USA, 79:2788-2792 (1982); and Sharma et al., J. Biol. Chem., 261:14160-14166 (1986). Possible counterparts to the bovine 59 and 61 kDa isozymes have also been isolated from rat tissues, Hansen et al., J. Biol. Chem., 261:14636-14645 (1986), suggesting that these two isozymes may be expressed in other mammalian species.

In addition to molecular weight criteria, other evidence supports both similarities and differences among the CaM-PDE family of isozymes. For example, the 59 kDa heart isozyme and the 61 kDa brain isozyme CaM-PDEs differ in mobility on SDS-PAGE and elution position on DEAE chromatography, and the 59 kDa isozyme has at least a 10-20 fold higher affinity for calmodulin. Oncomodulin, a fetal/onco calcium binding protein present in very high concentrations in the placenta and transformed cells, also binds to the 59 kDa enzyme with a higher affinity than to the 61 kDa enzyme. However, both

the 61 kDa brain and the 59 kDa heart isozymes are recognized by a single monoclonal antibody. This antibody binds to the Ca^{2+}/CaM-PDE complex with 100-fold higher affinity than to PDE alone. Hansen et al., 1986, supra. The 59 and 61 kDA isozymes have nearly identical substrate specificities and kinetic constants. Krinks et al., Adv. Cyc. Nucleotide Prot. Phosphorylation Res., 16:31-47 (1984) have suggested, based on peptide mapping experiments, that the heart 59 kDa protein could be a proteolytic form of the brain 61 kDa isozyme.

The 63 kDa bovine brain isozyme differs substantially from the 59 and 61 kDa isozymes. The 63 kDa enzyme is not recognized by the monoclonal antibody which binds to the 59 and 61 kDa enzymes. Hansen et al., 1986, supra. The 63 kDa protein is not phosphorylated in vitro by cAMP-dependent protein kinase, whereas the 61 kDa protein is phosphorylated. Further, only the 63 kDa protein is phosphorylated in vitro by CaM-kinase II. Sharma et al., Proc. Natl. Acad. Sci. (USA), 82:2603-2607 (1985); and Hashimoto et al., J. Biol. Chem., 264:10884-10887 (1989). The 61 and 63 kDa CaM-PDE isozymes from bovine brain do appear, however, to have similar CaM-binding affinities. Peptide maps generated by limited proteolysis with Staphylococcal V8 protease, Sharma et al., J. Biol.Chem., 259:9248 (1984), have suggested that the 61 and 63 kDa proteins have different amino acid sequences.

The cGMP-stimulated PDEs (cGS-PDEs) are proposed to have a noncatalytic, cGMP-specific site that may account for the stimulation of cAMP hydrolysis by cGMP. Stoop et al., J.Biol.Chem., 264:13718 (1989). At physiological cyclic nucleotide concentrations, this enzyme responds to elevated cGMP concentrations with an enhanced hydrolysis of cAMP. Thus, cGS-PDE allows for increases in cGMP concentration to moderate or inhibit

cAMP-mediated responses. The primary sequence presented recently in LeTrong et al., Biochemistry, 29:10280 (1990), co-authored by the inventors herein, provides the molecular framework for understanding the regulatory properties and domain substructure of this enzyme and for comparing it with other PDE isozymes that respond to different signals. This publication also notes the cloning of a 2.2kb bovine adrenal cortex cDNA fragment encoding cGS-PDE. See also, Thompson et al., FASEB J., 5(6):A1592 (Abstract No. 7092) reporting on the cloning of a "Type II PDE" from rat pheochromocytoma cells.

With the discovery of the large number of different PDEs and their critical role in intracellular signalling, efforts have focused on finding agents that selectively activate or inhibit specific PDE isozymes. Agents which affect cellular PDE activity, and thus alter cellular cAMP, can potentially be used to control a broad range of diseases and physiological conditions. Some drugs which raise cAMP levels by inhibiting PDEs are in use, but generally act as broad nonspecific inhibitors and have deleterious side effects on cAMP activity in nontargeted tissues and cell types. Accordingly, agents are needed which are specific for selected PDE isozymes. Selective inhibitors of specific PDE isozymes may be useful as cardiotonic agents, anti-depressants, anti-hypertensives, anti-thrombotics, and as other agents. Screening studies for agonists/antagonists have been complicated, however, because of difficulties in identifying the particular PDE isozyme present in a particular assay preparation. Moreover, all PDEs catalyze the same basic reaction; all have overlapping substrate specificities; and all occur only in trace amounts.

Differentiating among PDEs has been attempted by several different means. The classical enzymological

approach of isolating and studying each new isozyme is hampered by current limits of purification techniques and by the inability to accurately assess whether complete resolution of an isozyme has been achieved. A second approach has been to identify isozyme-specific assay conditions which might favor the contribution of one isozyme and minimize that of others. Another approach has been the immunological identification and separation into family groups and/or individual isozymes. There are obvious problems with each of these approaches; for the unambiguous identification and study of a particular isozyme, a large number of distinguishing criteria need to be established, which is often time consuming and in some cases technically quite difficult. As a result, most studies have been done with only partially pure PDE preparations that probably contained more than one isozyme. Moreover, many of the PDEs in most tissues are very susceptible to limited proteolysis and easily form active proteolytic products that may have different kinetic, regulatory, and physiological properties from their parent form.

The development of new and specific PDE-modulatory agents would be greatly facilitated by the ability to isolate large quantities of tissue-specific PDEs by recombinant means. Relatively few PDE genes have been cloned to date and of those cloned, most belong to the cAMP-specific family of phosphodiesterases (cAMP-PDEs). See Davis, "Molecular Genetics of the Cyclic Nucleotide Phosphodiesterases", pp. 227-241 in Cyclic Nucleotide Phosphodiesterases: Structure, Regulation, and Drug Action, Beavo, J. and Houslay, M.D., Eds.; John Wiley & Sons, New York; 1990. See also, e.g., Faure et al., PNAS (USA), 85:8076 (1988) - D. discoideum; Sass et al., PNAS (USA), 83:9303 (1986) - S. cerevisiae, PDE class IV, designated PDE2; Nikawa et al., Mol. Cell.

Biol., 7:3629 (1987) - S. cerevisiae, designated PDE1; Wilson et al., Mol. Cell. Biol., 8:505 (1988) - S. cerevisiae, designated SRA5; Chen et al., PNAS (USA), 83:9313 (1986) - D. melanogaster, designated dnc$^+$; Ovchinnikow et al., FEBS, 223:169 (1987) - bovine retina, designated GMP PDE; Davis et al., PNAS (USA), 86:3604 (1989) - rat liver, designated rat dnc-1; Colicelli et al., PNAS (USA), 86:3599 (1989) - rat brain, designated DPD; Swinnen et al., PNAS (USA), 86:5325 (1989) - rat testis, rat PDE1, PDE2, PDE3 and PDE4; and Livi et al., Mol. Cell. Biol., 10:2678 (1990) - human monocyte, designated hPDE1. See also, LeTrong et al., supra and Thompson et al., supra.

Complementation screening has been used to detect and isolate mammalian cDNA clones encoding certain types of PDEs. Colicelli et al., PNAS (USA), 86:3599 (1989), reported the construction of a rat brain cDNA library in an S. cerevisiae expression vector and the isolation therefrom of genes having the capacity to function in yeast to suppress the phenotypic effects of $RAS2^{val19}$, a mutant form of the RAS2 gene analogous to an oncogenic mutant of the human HRAS gene. A cDNA so cloned and designated DPD (rat dunce-like phosphodiesterase) has the capacity to complement or "rescue" the loss of growth control associated with an activated $RAS2^{val19}$ gene harbored in yeast strain TK161-R2V (A.T.C.C. 74050), as well as the analogous defective growth control phenotype of the yeast mutant 10DAB (A.T.C.C. 74049) which is defective at both yeast PDE gene loci (pde^{-1}, pde^{-2}). The gene encodes a high-affinity cAMP specific phosphodiesterase, the amino acid sequence of which is highly homologous to the cAMP-specific phosphodiesterase encoded by the dunce locus of Drosophila melanogaster.

Through the date of filing of parent application Serial No. 07/688,356, there have been no

reports of the cloning and expression of DNA sequences encoding any of the mammalian Ca^{2+}/calmodulin stimulated or cGMP-stimulated PDEs (PDE families I and II) and, accordingly, there continues to exist a need in the art for complete nucleotide sequence information for these PDEs.

BRIEF SUMMARY OF THE INVENTION

The present invention provides novel purified and isolated polynucleotide sequences (e.g. DNA and RNA including sense and antisense strands) which code for expression of mammalian species (e.g., human and bovine) Ca^{2+}/calmodulin stimulated cyclic nucleotide phosphodiesterase and cGMP stimulated cyclic nucleotide phosphodiesterase polypeptides. Genomic and cDNA sequences provided by the invention may be associated with homologous or heterologous species expression control DNA sequences such as promoters, operators, regulators, terminators and the like to allow for *in vivo* and *in vitro* transcription to messenger RNA and, in turn, translation of mRNAs to provide functional phosphodiesterases and related polypeptides in large quantities.

Specifically provided by the invention are mammalian DNA sequences encoding phosphodiesterases and fragments thereof which are present as mammalian DNA inserts in bacterial plasmids and viral vectors which are the subject of deposits made with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 on April 11 and 15, 1991 and on April 14, 1992 in accordance with U.S. Patent and Trademark Office and Budapest Treaty requirements. DNAs deposited in connection with the present invention include:

1. Plasmid pCAM-40 in *E. coli* (A.T.C.C. accession No. 68576) containing a bovine brain cDNA insert encoding a 61 kDa CaM-PDE isozyme;

2. Plasmid p12.3A in *E. coli* (A.T.C.C. 68577) containing a bovine brain cDNA insert encoding a 63 kDa CaM-PDE isozyme;

3. Bacteriophage λ CaM H6a (A.T.C.C. accession No. 75000) containing a human hippocampus cDNA insert fractionally encoding a 61 kDa CaM-PDE isozyme;

4. Plasmid pHcam61-6N-7 in E. coli (A.T.C.C. accession No. 68963) containing a composite human cDNA insert encoding a 61 kDa CaM-PDE isozyme;

5. Plasmid pcamH3EF in E. coli (A.T.C.C. accession No. 68964) containing a human hippocampus cDNA insert encoding a novel PDE homologous to a 61kDa CaM-PDE;

6. Plasmid pcamHella in E. coli (A.T.C.C. accession No. 68965) containing a human heart cDNA insert encoding a novel PDE homologous to a 61kDa CaM-PDE;

7. Plasmid p3CGS-5 in E. coli (A.T.C.C. accession No. 68579) containing a bovine adrenal cDNA insert encoding a cGS-PDE isozyme;

8. Plasmid pBBCGSPDE-5 in E. coli (A.T.C.C. accession No. 68578) containing a bovine brain cDNA insert encoding a cGS-PDE isozyme fragment;

9. Plasmid pBBCGSPDE-7 in E. coli (A.T.C.C. accession No. 68580) containing a bovine brain cDNA encoding a cGS-PDE isozyme;

10. Plasmid pGSPDE6.1 in E. coli (A.T.C.C. accession No. 68583) containing a human heart cDNA encoding a cGS-PDE isozyme fragment;

11. Plasmid pGSPDE7.1 in E. coli (A.T.C.C. accession No. 68585) containing a human hippocampus cDNA insert encoding a cGS-PDE isozyme fragment; and

12. Plasmid pGSPDE9.2 (A.T.C.C. accession No. 68584) containing a human hippocampus cDNA insert encoding a cGS-PDE isozyme fragment.

13. Plasmid pHcgs6n in E. coli (A.T.C.C. accession No. 68962) containing a human cDNA insert encoding a cGS-PDE.

Also specifically provided by the present invention is a bovine cDNA sequence containing nucleotides encoding bovine 59 kDa CaM-PDE and characterized by the DNA and amino acid sequences of SEQ ID NO: 16 and SEQ ID NO: 17.

In related embodiments, the invention concerns DNA constructs which comprise a transcriptional promoter, a DNA sequence which encodes the PDE or a fragment thereof, and a transcriptional terminator, each operably linked for expression of the enzyme or enzyme fragment. The constructs are preferably used to transform or transfect host cells, preferably eukaryotic cells, and more preferably mammalian or yeast cells. For large scale production, the expressed PDE can be isolated from the cells by, for example, immunoaffinity purification.

Incorporation of DNA sequences into procaryotic and eucaryotic host cells by standard transformation and transfection processes, potentially involving suitable DNA and RNA viral vectors and circular DNA plasmid vectors, is also within the contemplation of the invention and is expected to provide useful proteins in quantities heretofore unavailable from natural sources. Systems provided by the invention include transformed E. coli cells, including those referred to above, as well as other transformed eukaryotic cells, including yeast and mammalian cells. Use of mammalian host cells is expected to provide for such post-translational modifications (e.g., truncation, lipidation, and tyrosine, serine or threonine phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products of the invention.

Novel protein products of the invention include expression products of the aforementioned nucleic acid sequences and polypeptides having the primary structural conformation (i.e., amino acid sequence) of CaM-PDE and

cGS-PDE proteins, as well as peptide fragments thereof and synthetic peptides assembled to be duplicative of amino acid sequences thereof. Proteins, protein fragments, and synthetic peptides of the invention are projected to have numerous uses including therapeutic, diagnostic, and prognostic uses and will provide the basis for preparation of monoclonal and polyclonal antibodies specifically immunoreactive with the proteins of the invention.

Also provided by the present invention are antibody substances (including polyclonal and monoclonal antibodies, chimeric antibodies, single chain antibodies and the like) characterized by their ability to bind with high immunospecificity to the proteins of the invention and to their fragments and peptides, recognizing unique epitopes which are not common to other proteins. The monoclonal antibodies of the invention can be used for affinity purification of CaM-PDEs and cGS-PDEs, e.g., Hansen et al., Meth. Enzymol., 159:543 (1988).

Also provided by the present invention are novel procedures for the detection and/or quantification of normal, abnormal, or mutated forms of CaM-PDEs and cGS-PDEs, as well as nucleic acids (e.g., DNA and mRNA) associated therewith. Illustratively, antibodies of the invention may be employed in known immunological procedures for quantitative detection of these proteins in fluid and tissue samples, and of DNA sequences of the invention that may be suitably labelled and employed for quantitative detection of mRNA encoding these proteins.

Among the multiple aspects of the present invention, therefore, is the provision of (a) novel CaM-PDE and cGS-PDE encoding polynucleotide sequences, (b) polynucleotide sequences encoding polypeptides having the activity of a mammalian CaM-PDE or of a mammalian cGS-PDE which hybridize to the novel CaM-PDE and cGS-PDE encoding

sequences under hybridization conditions of the stringency equal to or greater than the conditions described herein and employed in the initial isolation of cDNAs of the invention, and (c) polynucleotide sequences encoding the same (or allelic variant or analog polypeptides) through use of, at least in part, degenerate codons. Correspondingly provided are viral DNA and RNA vectors or circular plasmid DNA vectors incorporating polynucleotide sequences and procaryotic and eucaryotic host cells transformed or transfected with such polynucleotide sequences and vectors, as well as novel methods for the recombinant production of these proteins through cultured growth of such hosts and isolation of the expressed proteins from the hosts or their culture media.

In yet other embodiments, the invention provides compositions and methods for identifying compounds which can modulate PDE activity. Such methods comprise incubating a compound to be evaluated for PDE modulating activity with eukaryotic cells which express a recombinant PDE polypeptide and determining therefrom the effect of the compound on the phosphodiesterase activity provided by gene expression. The method is effective with either whole cells or cell lysate preparations. In a preferred embodiment, the eukaryotic cell is a yeast cell or mammalian cell which lacks endogenous phosphodiesterase activity. The effect of the compound on phosphodiesterase activity can be determined by means of biochemical assays which monitor the hydrolysis of cAMP and/or cGMP, or by following the effect of the compound on the alteration of a phenotypic trait of the eukaryotic cell associated with the presence or absence of the recombinant PDE polypeptide.

Other aspects and advantages of the present invention will be apparent upon consideration of the

following detailed description thereof which includes numerous illustrative examples of the practice of the invention, reference being mad to the drawing wherein:

Figure 1 provid s the results of amino acid sequence determinations for isolated 59 kDa (bovine heart) and 63 kDa (bovine brain) CaM-PDE proteins in alignment with the complete sequence of the 61 kDa (bovine brain) isozyme. Identities of the 59 and 63 kDa proteins to the 61 kDa isozyme are underlined. Tentative identifications are in lower cases and hyphens denote unidentified residues. The N-terminus of the 59 kDa isozyme, as determined by the subtraction of a methionyl peptide (mDDHVTIRRK) from the composition of an amino-terminal blocked lysyl peptide, is in parenthesis. Solid boxes are placed above residues within the CaM-binding sites identified in the 61 and 59 kDa isozymes.

DETAILED DESCRIPTION OF THE INVENTION

The following examples illustrate practice of the invention. Example I relates to the isolation, purification, and sequence determination of 61 kDa CaM-PDE cDNA from bovine brain and to the expression thereof in a mammalian host cell. Example II relates to the isolation, purification, and sequence determination of a 59 kDa CaM-PDE from bovine lung and to the expression thereof in a mammalian host cell. Example III relates to the isolation, purification, and sequence determination of 63 kDa CaM-PDE cDNA from bovine brain and to the expression thereof in a mammalian host cell. Example IV relates to the isolation, purification, and sequence determination of cGS-PDE cDNA from bovine adrenal cortex, as well as the expression of the DNA in mammalian host cells. Example V relates to the isolation, purification, and sequence determination of cGS-PDE cDNA from bovine brain and to the expression thereof in a mammalian host cell. Example VI relates to the use of cGS-PDE bovine adrenal cDNA to obtain human cGS-PDE cDNAs and to the development of a human cDNA encoding a cGS-PDE. Example VII relates to the use of CaM-PDE 61 kDa bovine brain cDNA to obtain a human CaM-PDE 61 kDa cDNA and a novel structurally related cDNA. Example VIII relates to the expression of bovine and human PDE cDNAs for complementation of yeast phenotypic defects and verification of phosphodiesterase activity for the expression product. Example IX relates to tissue expression studies involving Northern analysis and RNase protection studies employing polynucleotides (specifically cDNAs and antisense RNAs) of the invention.

In those portions of the text addressing the formation of redundant oligonucleotides, the following Table I single letter code recommendations for ambiguous

nucleotide sequence, as reported in J.Biol.Chem., 261:13-17 (1986), are employed:

TABLE I

Symbol	Meaning	Origin of designation
G	G	Guanine
A	A	Adenine
T	T	Thymine
C	C	Cytosine
R	G or A	puRine
Y	T or C	pYrimidine
M	A or C	aMino
K	G or T	Keto
S	G or C	Strong interaction (3 H bonds)
W	A or T	Weak interaction (2 H bonds)
H	A, C, or T	not G, as H follows G in the alphabet
B	G, C, or T	not A
V	A, C, or G	not T, (not U) as V follows U
D	A, G, or T	not C
N	A, C, G, or T	any Nucleotide base

EXAMPLE I

Isolation, Purification, and Sequence Determination of 61 kDa CaM-PDE cDNA From Bovine Brain

In this Example, a cDNA sequence representing that portion of a gene for 61 kDa bovine brain CaM-PDE which encodes the amino terminus of the protein was isolated by PCR from a collection of first strand cDNAs

developed from bovine brain mRNA. The PCR-generated fragment was then employed to isolate a full length bovine brain CaM-PDE sequence.

Total RNA was prepared from bovine heart using the method of Chomczynski et al., Anal.Biochem., 162:156-159 (1987) and mRNA was selected using a Poly(A) QuikTm mRNA purification kit according to the manufacturer's protocol. First strand cDNA was synthesized by adding 80 units of AMV reverse transcriptase to a reaction mixture (40 µl, final volume) containing 50 mM Tris HCl (pH8.3 @ 42°), 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM (each) deoxynucleotide triphosphates, 50 mM KCl, 2.5 mM sodium pyrophosphate, 5 µg deoxythymidylic acid oligomers (12-18 bases) and 5 µg bovine heart mRNA denatured for 15 min at 65°. Incorporation of 1 µl [^{32}P]-labeled dCTP (3000 Ci/mmol) was used to quantitate first strand cDNA synthesis. The reaction was incubated at 42° for 60 min. The reaction was phenol/$CHCl_3$ extracted and EtOH precipitated. The nucleic acid pellet was resuspended in 50 µl of 10 mM Tris-HCl (pH 7.5)/0.1 mM EDTA to a final concentration of 15 ng per µl.

Redundant sense and antisense oligomers corresponding to 61 kDa peptide sequences as in Fig. 1 were designed to be minimally redundant, yet long enough to specifically hybridize to the target template.

A first 23 base oligomer, designated CaM PCR-2S, was synthesized on an Applied Biosystems, Inc. DNA synthesizer. The oligomer had the following sequence,

SEQ ID NO: 1

5'-AARATGGGNATGAARAARAA-3'

which specifies the following amino acid sequence,

SEQ ID NO: 2
KMGMMKKK.

A second 23 base oligomer, designated CaM PCR-3AS, was synthesized with the following sequence,

SEQ ID NO: 3
5'-ACRTTCATYTCYTCYTCYTGCAT-3'

representing the following amino acid sequence,

SEQ ID NO: 4
MQEEEMNV.

A 612 bp CaM PDE cDNA fragment was synthesized using the PCR amplification technique by adding 15 ng of first strand cDNA to a reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 0.2 mM (each) deoxynucleotide triphosphates, 1 μM (each) CaM PCR 2S and CaM PCR-3AS oligomers, and 2.5 units of Thermus aquaticus DNA polymerase. The reaction was incubated for 30 cycles as follows: 94° for 1 min; 50° for 2 min; and 72° for 2 min. The reaction products were purified on a 1% agarose gel using 0.04 M Tris-acetate/0.001 M EDTA buffer containing 0.5 μg/ml ethidium bromide. The DNA products were visualized with UV light, cleanly excised from the gel with a razor blade, purified using Geneclean II reagent kit and ligated into Eco RV-cut pBluescript vector DNA.

To determine if the PCR amplification products were CaM PDE cDNAs, the subcloned PCR DNA products were sequenced from the ends using T3 and T7 promoter primers and either Sequenase or Taq Polymerase sequencing kits. Approximately 250 bases from each end of this piece of

DNA were sequenced and the deduced amino acid sequence from the cDNA corresponded with the Fig. 1 amino acid sequences of the 59 and 61 kDa CaM-PDEs, confirming that the PCR DNA product was a partial CaM PDE cDNA.

A bovine brain cDNA library constructed with the lambda ZAP vector (kindly provided by Ronald E. Diehl, Merck, Sharp & Dohme) was screened with the radiolabeled 615 bp CaM-PDE cDNA obtained by PCR amplification. The probe was prepared using the method of Feinberg et al., Anal.Biochem., 137:266-267 (1984), and the [^{32}P]-labeled DNA was purified using Elutip-D® columns. Plaques (700,000 plaques on 12-150 mm plates) bound to filter circles were hybridized at 42°C overnight in a solution containing 50% formamide, 20 mM Tris-HCl (pH 7.5), 1X Denhardt's solution, 10% dextran sulfate, 0.1% SDS and 10^6 cpm/ml [^{32}P]-labeled probe (10^9 cpm/μg). The filters were washed three times for 15 min with 2X SSC/0.1% SDS at room temperature, followed by two 15-min washes with 0.1X SSC/0.1% SDS at 45°C. The filters were exposed to x-ray film overnight.

Of the fifty-six plaques that hybridized with the [^{32}P]-labeled probes eight randomly selected clones were purified by several rounds of re-plating and screening [Maniatis et al., Molecular Cloning: A Laboratory Manual 545 pp. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., (1982)] and the insert cDNA's were subcloned into pBluescript SK(-) by in vivo excision [Short et al., Nuc. Acids Res., 16:7583-7599 (1988)] as recommended by the manufacturer.

Plasmid DNA prepared from cultures of each clone were subjected to restriction analysis using EcoRI. Two clones of a suitable length were selected for sequence analysis using Taq Tak® and Sequenase® sequencing kits. The two clones were pCAM-40 (2.3kb) and pCAM-34 (2.7kb). The sequencing information from this

procedure confirmed that the insert of pCAM-40 encoded the full length bovine brain 61 kDa CaM-PDE. The sequence of this clone and the amino acid sequence deduced therefrom are set forth in SEQ ID NO: 5 and SEQ ID NO: 6.

Transient expression of the 61kDa CaM-PDE cDNA in COS-7 cells (A.T.C.C. CRL 1651) was accomplished as follows. Vector pCDM8 [Seed, Nature, 329:840-843 (1987)] in E. coli host cells MC1061-p3 was generously provided by Dr. Brian Seed, Massachusetts General Hospital, Boston, MA. This vector is also available from Invitrogen, Inc. (San Diego, CA). Plasmid pCAM-40 was digested with HindIII and NotI, yielding a 2.3kb fragment which was ligated into CDM8 vector DNA which had been digested with HindIII and NotI. The resulting plasmid was propagated in MC1061-p3 cells. Plasmid DNA was prepared using the alkaline lysis method of Ausubel et al., eds., Current Protocols in Molecular Biology, 1:1.7.1 (John Wiley & Sons, New York, 1989) and purified using Qiagen-Tip 500 columns (Qiagen, Inc. Chatsworth, CA) according to the manufacturer protocol.

COS-7 cells were transfected with the p-CAM-40/CDM8 construct (or mock transfected with the CDM8 vector alone) using the DEAE-dextran method Ausubel et al., supra at 1:9.2 et seq. Specifically, 10 μg of ethanol precipitated DNA was resuspended in 80 μl TBS buffer, and added to 160 μl of 10 mg per ml DEAE-dextran dropwise to a 100 mm plate of 50% confluent COS-7 cells in 4 ml of DMEM supplemented with 10% NuSerum, and mixed by swirling. The cells were incubated for 3-4 hours at 37° in a water-saturated 7% CO_2 atmosphere. The medium was removed and the cells were immediately treated with 10% DMSO in PBS for 1 minute. Following this treatment, the cells were washed with PBS, then DMEM, and finally cultured in DMEM supplemented with 10% fetal bovine serum

and antibiotics (50 μg/ml streptomycin sulfate) in a 7%-CO_2 incubator for 36 hours.

COS cells were scraped from the plates and homogenized in a buffer containing 40 mM Tris-HCl (pH=7.5), 5 mM EDTA, 15 mM benzamidine, 15 mM beta-mercaptoethanol, 1 μg per ml pepstatin A and 1 μg per ml peupeptin using a Dounce homogenizer (1 ml per 100 mm plate). Homogenates were assayed for PDE activity according to the procedures of Hanson et al., Proc. Nat'l. Acad. Sci., U.S.A., 79:2788-2792 (1982), using [^{3}H]cGMP as the substrate. Reactions were carried out at 30° for 10 minutes in a buffer containing 20 mM Tris-HCl (pH=7.5), 20 mM imidazole (pH=7.5), 3 mM $MgCl_2$, 15 mM Mg acetate, 0.2 mg per ml BSA and 1 μM ^{3}H-cAMP with either 2 mM EGTA or 0.2 mM $CaCl_2$ and 4 μg per ml CaM. Assays were stopped by incubating the tubes in a 90° water bath for 1 minute. After cooling, 10 μl of 2.5 mg per ml snake venom was added to each assay and incubated at 37° for 5 minutes. The samples were diluted with 250 μl of 20 mM Tris-HCl (pH=7.5) and immediately applied to 0.7 ml A-25 ion exchange columns. The columns were washed three times with 0.5 ml of 20 mM Tris-HCl (pH=7.5) and the eluate was collected in scintillation vials. Samples were counted for 1 minute using a Packard Model 1600TR scintillation counter. Specific cyclic nucleotide hydrolytic activity was expressed as picomoles cAMP or cGMP hydrolyzed per minute per mg protein. Protein concentration was estimated according to the method of Bradford, Anal. Biochem., 72:248-254 (1976), using BSA as a standard. When compared to mock transfected cells, extracts of cells transfected with pCAM-40 cDNA contained significantly greater CAMP and cGMP hydrolytic activities in the presence of EGTA. Assays of the pCAM-40 cDNA-transfected cells in the presence of calcium and CaM resulted in stimulation of cAMP and cGMP hydrolysis.

EXAMPLE II

Isolation, Purification, and Sequence Determination of a 59 kDa CaM-PDE From Bovine Lung

A fully degenerate sense oligonucleotide corresponding to the amino acid sequence

SEQ ID NO: 7
MDDHVTI

from the bovine heart 59 kDa CaM-pde was synthesized. The nucleotide sequence of this oligonucleotide is

SEQ ID NO: 8
5'-ATGAGRAGRCAYGTHACNAT-3'.

An antisense oligonucleotide was designed from the Fig. 1 sequence of bovine brain 61 kDa CaM-PDE, corresponding to the amino acid sequence

SEQ ID NO: 9
LRCLVKQ

and having the sequence,

SEQ ID NO: 10
5'-CTGCTTCACTAAGCATCTTAG-3'.

This primer pair was used to prime a PCR reaction using bovine heart first strand cDNA (as prepared in Example I) as a template. This predicted a PCR product of 75 bp, 54 bp of which were unique 59 kDa sequence and 21 bp of which were shared between the 59 kDa and 61 kDa isozymes. The PCR products were analyzed by sieving agarose gel electrophoresis, and a band migrating at 75 bp was

excised from the gel. The DNA was subcloned into pBluescript KS*, and colonies positive by the blue/white selection scheme were screened by PCR using primers directed against vector sequences. Colonies with inserts of the appropriate size were selected, and one of these (pCaM59/75.14) was chosen for sequencing. Plasmid DNA was prepared using a Qiagen P20 push column and used as template for sequencing using the dideoxy method. The sequence of the PCR product is

SEQ ID NO: 11

5'-ATGAGAAGGCACGTAACGATCAGGAGGAAACATCTCCAA
AGACCCATCTTT-AGACTAAGATGCTTAGTGAAGCAG-3'.

Analysis of the sequence revealed differences in two codons between the sequence obtained and the predicted sequence. Re-examination of the sense oligonucleotide primer sequence revealed that an inadvertent transposition of two codons had led to a mistake in the design of the oligonucleotide. A second set of oligonucleotide PCR primers was prepared which predicted a 54 bp product with minimum overlap between the 59 and 61 kDa isozymes; in addition, the second sense primer incorporated a correction of the mistake in the design of the original sense primer. The sense oligonucleotide had the sequence

SEQ ID NO: 12

5'-ATGGAYGAYCACGTAACGATC-3'

and the antisense oligonucleotide had the sequence

SEQ ID NO: 13

5'-AAGTATCTCATTGGAGAACAG-3'

This primer pair was used to prime a PCR reaction using bovine heart first-strand cDNA as template and the PCR products subcloned and screened exactly as described above. Two clones (pCaM59/54.9 and pCaM59/54.10) were selected for sequencing based on insert size and sequenced as described above; both clones contained 54 bp inserts of the predicted sequence

SEQ ID NO: 14

5'-ATGGATGATCACGTAACGATCAGGAGGAAACATCTCCAAA GACCCATCT-TTAGA-3',

predicting the amino acid sequence

SEQ ID NO: 15

MDDHVTIRRKHLQRPIFR

A cDNA library was constructed from bovine lung mRNA and screened using procedures as described in Example IV, infra, with respect to screening of a bovine adrenal cortex library. Approximately 1.2×10^6 plaque-forming units were probed with a ^{32}P-labelled, 1.6kb EcoRI restriction endonuclease-cleavage product of the pCAM-40 cDNA. This initial screening produced 4 putative 59 kDA CaM-PDE cDNA clones. Preliminary sequence analysis indicated that one clone, designated p59KCAMPDE-2, contained the complete coding sequence of the putative 59 kDa CaM-PDE. A series of nested deletions were constructed from the p59KCAMPDE-2 plasmid [See, Sonnenburg et al., J. Biol. Chem., 266 (26): 17655-17661 (1991)], and the resultant templates were sequenced by an adaptation of the method of Sanger using the Taq DyeDeoxy™ Terminator Cycle Sequencing Kit and an Applied Biosystems Model 373A DNA Sequencing System. The DNA and deduced amino acid sequences are set out in SEQ. ID NO:

16 and 17, respectively. A large open reading frame within the cDNA encodes a 515 residue polypeptide with an estimated molecular weight of ≈ 59 kilodaltons that is nearly identical to the 61 kDa CaM-PDE amino acid sequence except for the amino-terminal 18 residues. Moreover, the predicted amino acid sequence of the p59KCAMPDE-2 open reading frame is identical to the available sequence of the 59 kDa CaM-PDE purified from bovine heart, Novack et al., Biochemistry, 30: 7940-7947 (1991). These results indicate that the p59KCAMPDE-2 cDNA represents an mRNA species encoding the 59 kDa CaM-PDE.

Transient expression of the 59 kDa bovine lung PDE was accomplished as in Example I. Specifically, a 2.66kb, EcoRI/blunt-ended fragment of p59KCAMPDE-2 cDNA was subcloned into pCDM8 which had been digested with XhoI and blunt-ended. The recombinant plasmid, designated p59KCAMPDE-2/CDM8, was used to transiently transfect COS-7 cells and extracts prepared from transfected COS-7 cells were assayed for CaM-PDE activity using 2 μM cAMP. COS-7 cells transfected with the p59KCAMPDE-2 cDNA yielded a cAMP hydrolytic activity that was stimulated 4-5 fold in the presence of calcium and calmodulin. Mock transfected COS-7 cells had no detectable calmodulin-stimulated cAMP hydrolytic activity.

EXAMPLE III

Isolation, Purification, and Sequence Determination of 63 kDa CaM-PDE cDNA From Bovine Brain

Multiple fully and partially redundant oligonucleotides corresponding to the amino acid sequence reported in Fig. 1 were synthesized for use in attempting to obtain a cDNA clone for the 63 kDa CaM-PDE. Annealing

temperatures used for the polymerase chain reactions were varied between 2 to 20°C below the theoretical melting temperature for the lowest melting oligonucleotide of each sense-antisense pair. Except for probes 63-12s and 63-13a, which are discussed below, the PCR products of each of the oligonucleotide pairs under a wide range of conditions gave multiple ethidium bromide bands when agarose gel-electrophoresed. Use of 63-12s and 63-13a resulted in a PCR product that coded for 63 kDa CaM-PDE when sequenced.

A fully redundant sense 23-mer oligonucleotide, designated 63-12s, was assembled having the following sequence

SEQ ID NO: 18

5'ATHCAYGAYTAYGARCAYACNGG-3'

based on an amino acid sequence,

SEQ ID NO: 19

IHDYEHTG

which is conserved in the 61 kDa bovine CaM-PDEs (see Fig. 1). A partially redundant antisense 32-mer oligonucleotide, designated 63-13a, had the sequence

SEQ ID NO: 20

5'-TCYTTRTCNCCYTGNCGRAARAAYTCYTCCAT-3'

and was based on the following conserved sequence in the 63 kDa CaM-PDE,

SEQ ID NO: 21

MEEFFRQGDKE

Messenger RNA was prepared from bovine brain cerebral cortex and poly A$^+$ selected. First strand complementary DNA was produced using AMV or MMLV reverse transcriptase. De-tritylated oligonucleotides were phosphorylated using 1mM [γ-^{32}P]ATP at 1 X 10^6 cpm/nmol and T4 polynucleotide kinase. After separation of 5′ ^{32}P-labelled oligonucleotides from free ATP using NENsorb 20 columns, each was suspended as a 20 μM (5′ phosphate) stock and combined finally at 400 nM each in the PCR. The reaction was run using 50 ng total cDNA and 200 μM dNTP to obtain about 1 μg of PCR product. The reaction had an initial denaturation step at 94°C for 5 min followed by 30 cycles of a 1 min 94°C denaturation, an annealing step at 50°C for 1 min, and a 2 min extension step at 72°C. Under the reaction conditions, a single ethidium bromide-staining band of 450 base pairs was obtained on agarose gel electrophoresis of 100 ng of the PCR product. Five μg of 5′ phosphorylated PCR product was ligated to 15 ng EcoRV-cut Bluescript KS(+) plasmid using T4 DNA ligase in 5% PEG-6000 for 12 h at 21°C. Putative positives of XL 1-blue transformations were white colonies using isopropyl thiogalactoside (IPTG) and bromo- chloro- indolyl galactoside (Xgal) for chromogenic selection. Such picks were sequenced using T3 or T7 primers, dideoxynucleotide terminators, and Sequenase.

One resultant clone (p11.5B) had the nucleotide sequence and translated amino acid sequence provided in SEQ ID NO: 22 and SEQ ID NO: 23, respectively. The codons for the amino acids YEH found in oligonucleotide 63-12s were replaced by codons for the amino acid sequence NTR in p11.5B. This was probably due to a contaminant in 63-12s. Since the translated open reading frame (ORF) was similar to that reported in Fig. 1 for the 63 kDa CaM PDE, p11.5B was used to screen a bovine brain cDNA library for a full length cDNA clone.

A bovine brain cDNA library was constructed in λ ZAP II. First strand cDNA was treated with RNase H, E. coli DNA polymerase, and E. coli DNA ligase to synthesize second strand cDNA. The cDNA was blunt-ended by T4-DNA polymerase; EcoRI sites in the cDNA were protected with EcoRI methylase and S-adenosyl methionine and EcoRI linkers were ligated on with T4 DNA ligase. After EcoRI restriction endonuclease treatment, free linkers were separated from the cDNA by gel filtration over Sepharose CL-4B. λ ZAP II arms were ligated onto the cDNA and packaged by an in vitro Gigapack Gold packaging kit obtained from Stratagene. 9.5×10^5 recombinants were obtained with 5.8% nonrecombinant plaques as assessed by plating with IPTG and X-gal. The library was amplified once by the plate lysate method to obtain 1.4×10^7 pfu/ml.

An initial screen of a total bovine brain cDNA library in λ ZAP II was performed. 700,000 pfu were screened using ^{32}P-labelled oligonucleotide 63-1s at a hybridization and wash temperature of 40°C. Oligonucleotide 63-1s was a fully redundant 23-mer having the sequence

SEQ ID NO: 24
5'-AARAARAAYYTNGARTAYACNGC-3'

corresponding to the amino acid sequence

SEQ ID NO: 25
KKNLEYTA

A total of 21 putative positives were picked. Subsequent rescreens were impeded by the very high background found using this screening method. Therefore, aliquots of each primary pick were pooled and 50,000 pfu of the pool were

replated and rescreened with p11.5B radiolabelled by random primers and [α-^{32}P]dCTP. One positive was obtained, plaque-purified, and rescued as a plasmid p12.3a. Its DNA sequence is provided in SEQ ID NO: 26. Subsequently, the bovine brain cerebral cortex library was screened further with p11.5B. Two further independent clones, p12.27.9 and p12.27.11, were obtained out of a primary screen of 1.4 X 10^6 pfu. They were plaque-purified and rescued for sequencing.

Clone p12.3a codes for a protein sequence with most of the aligned peptides isolated from bovine 63 kDa CaM-PDE as shown in Fig. 1. SEQ ID NO: 26 and SEQ ID NO: 27 set forth the coding region (i.e., the 1844 nucleotides of an approximately 2.5 kilobase insert) of p12.3a. Base numbers 248-290 code for amino acid sequence

SEQ ID NO: 28
QLENGEVNIEELKK,

while the comparable (Figure 1) peptide has the sequence

SEQ ID NO: 29
QLIPGRVNIISLKK

Base numbers 942-990 code for an amino acid sequence

SEQ ID NO: 30
KSECAILYNDRSVLEN

while the isolated (Figure 1) peptide sequence is

SEQ ID NO: 31
KDETAILYNDRTVLEN.

None of the nonaligned 63 kDa peptide sequence is found in any reading frame of p12.3a; also, the molecular weight of the p12.3a open reading frame, as translated, is 60,951 not 63,000. Therefore, this cDNA may represent an isozyme variant of the 63 kDa protein. The other two independent clones (p12.27.9 and p12.27.11) seem to have ORF sequence identical to p12.3a. The open reading frame of one clone begins at nucleotide number 823 of p12.3a and is identical to p12.3a through its termination codon. The other clone starts at nucleotide 198 and is identical to p12.3a throughout its length. None of the three clones has the anomalous NTR peptide sequence found in p11.5B; all three have YEH as the 61 kDa CaM PDE.

Transient expression of the 63kDa CaM-PDE cDNA in COS-7 cells was accomplished as follows. A fragment of the cDNA insert of plasmid p 12.3 including the protein coding region of SEQ.ID NO: 26 and flanked by BamHI restriction sites was prepared by PCR. More specifically, oligonucleotides corresponding to base Nos. 94-117 (with the putative initiation codon) and the antisense of base Nos. 1719-1735 (with sequence immediately 3′ of the termination codon) of SEQ.ID NO. 26 were synthesized with two tandem BamHI sites on their 5′ ends. The two primers had the following sequences:

SEQ.ID NO: 32

5′-GGATCCGGATCCCGCAGACGGAGGCTGAGCATGG-3′

SEQ.ID NO: 33

5′-GGATCCGGATCCAGGACCTGGCCAGGCCCGGC-3′

The two oligonucleotides were used in a PCR cycling 30 times from a 1 min incubation at 94°C to a 2 min 72°C incubation with a final 10 min extension reaction at 72°C. The 100 µl reaction used 20 µM of each

oligonucleotide and 100 pg p12.3a as the template in order to produce 5 μg 1665 base pair product.

The product was extracted once with an equal volume of 1:1 phenol:chloroform, made 0.3 M with regard to sodium acetate, and precipitated with two volumes of ethanol overnight. The precipitate was dried, rehydrated into 50 μl, and the cDNA was digested with 5 units BamHI restriction endonuclease for one hour at 37°C. Afterwards, the solution was extracted once with an equal volume of 1:1 phenol:chloroform. The 1641 base pair cDNA with BamHI 5′ and 3′ ends was purified from the aqueous layer using Qiagen Q-20 columns (Qiagen, Inc., Chatsworth, CA) and the protocol given by the manufacturer.

The cut, purified PCR product was ligated into BamHI digested, alkaline phosphatase-treated Bluescript KS(+) plasmid. The ligation product was subcloned into XL1 cells; resulting transformants were screened by sequencing. One transformant (designated p11.6.c6) was isolated with the BamHI insert oriented such that the Bluescript KS(+) HindIII restriction site was 30 bases 5′ to the sequence of the insert encoding the initiation codon. This plasmid was digested with HindIII and XbaI restriction endonucleases to release the 1689 base pair fragment. The fragment was ligated into HindIII- and XbaI-digested CDM8 vector DNA as in Example I.

COS-7 cells were transfected with the p12.3.a/CDM8 construct or mock transfected with the CDM8 vector alone using the DEAE-dextran method as described in Example 1. A ratio of 10 μg DNA/400 μg DEAE-dextran was used, with a final DEAE-dextran concentration in the media of 100 μg/ml. After 48 h, cells were suspended in 1 ml of homogenization buffer (40 mM Tris HCl, pH=7.5, 15 mM benzamidine HCl, 15 mM β-mercaptoethanol, 0.7 μg/ml pepstatin A, 0.5 μg/ml leupeptin, and 5 mM Na_4EDTA) and

disrupted on ice using a Dounce homogenizer. The homogenates were diluted 1/2 to make a final 50% (v/v) glycerol for storag at -20°C and used either to assay for phosphodiesterase activity or to determine protein concentration. CaM-dependent and independent activities were determined as in Example 1. Cells transfected with a p12.3.a DNA had a 15-fold increase in CaM-stimulated cAMP phosphodiesterase activity and a 12-fold increase in CaM-stimulated cGMP phosphodiesterase activity over basal levels. Mock transfected COS-7 cells showed no PDE activity over basal levels even with CaM stimulation.

EXAMPLE IV

Isolation, Purification, Sequence Determination, and Expression of cGS-PDE cDNA From Bovine Adrenal Cortex

Total RNA was prepared from bovine adrenal outer cortex using the method of Chomczynski et al., supra. Polyadenylated RNA was purified from total RNA preparations using the Poly(A) QuickTm mRNA purification kit according to the manufacturer's protocol. First strand cDNA was synthesized by adding 80 units of AMV reverse transcriptase to a reaction mixture (40 µl, final volume) containing 50 mM Tris-HCl (pH 8.3 @ 42°), 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM (each) deoxynucleotide triphosphates, 50 mM KCl, 2.5 mM sodium pyrophosphate, 5 µg deoxythymidylic acid oligomers (12-18 bases) and 5 µg bovine adrenal cortex mRNA denatured for 15 min at 65°C. The reaction was incubated at 42°C for 60 min. The second strand was synthesized using the method of Watson et al., DNA Cloning: A Practical Approach, 1:79-87 (1985) and the ends of the cDNA were made blunt with T4 DNA polymerase. EcoRI restriction endonuclease sites were methylated [Maniatis et al., supra] using a EcoRI methylase (Promega), and EcoRI linkers (50-fold molar

excess) were ligated to the cDNA using T4 DNA ligase. Excess linkers were removed by digesting the cDNA with EcoRI restriction endonuclease, followed by Sepharose CL-4B chromatography. Ausubel et al., supra. The cDNA (25-50 ng per μg vector) was ligated into EcoRI-digested, dephosphorylated ZAP® II (Stratagene) arms [Short et al., Nuc.Acids Res., 16:7583-7599 (1988)] and packaged [Maniatis et al., supra] with Gigapack® Gold extracts according to the manufacturer's protocol.

Initially, an unamplified bovine adrenal cortex cDNA library was made and screened with a redundant 23-mer antisense oligonucleotide probes end-labeled with γ-[^{32}P]ATP and T4 polynucleotide kinase. The oligomers corresponding to the amino acid sequences

SEQ ID NO: 34
EMMMYHMK

and

SEQ ID NO: 35
YHNWMHAF

were made using an Applied Biosystems model 380A DNA synthesizer. Their sequences are as follows:

SEQ ID NO: 36
5'-TT CAT RTG RTA CAT CAT CAT YTC-3'

SEQ ID NO: 37
5'-AA NGC RTG CAT CCA RTT RTG RTA-3'

Duplicate nitrocellulose filter circles bearing plaques from 12 confluent 150 mm plates (approximately 50,000 pfu/plate) were hybridized at 45°C overnight in a

solution containing 6X SSC, 1X Denhardt's solution, 100 μg/ml yeast tRNA, 0.05% sodium pyrophosphate and 10^6 cpm/ml radiolabeled probe (>10^6 cpm per pmol). The filters were washed three times in 6X SSC at room temperature, followed by a higher-stringency 6X SSC wash at 10°C below the minimum melting temperature of the oligomer probes, and exposed to x-ray film overnight.

A single 2.1 kb cDNA clone (designated pcGS-3:2.1) was isolated and sequenced. The amino acid sequence enclosed by the large ORF of this clone was identical to peptide sequences of the cGS-PDE purified from the supernatant fraction of a bovine heart homogenate. LeTrong et al., supra.

A second, amplified, bovine adrenal cortex cDNA library was screened using the [^{32}P]-labeled CGS-3:2.1 partial cDNA, yielding a 4.2 kb cDNA (designated 3CGS-5).

The library was constructed, amplified as in Maniatis et al., supra, plated and screened with the bovine cDNA insert from clone CGS-3:2.1. The probe was prepared using the method of Feinberg et al., supra, and the radiolabeled DNA was purified using Elutip-D® columns. Plaques (600,000 pfu on twelve 150 mm plates) bound to filter circles were hybridized at 42°C overnight in a solution containing 50% formamide, 20 mM Tris-HCl (pH 7.5, 1X Denhardt's solution, 10% dextran sulfate, 0.11% SDS and 10^6 cpm/ml [^{32}P]-labeled probe (10^9 cpm/μg). The filters were washed three times for 15 minutes with 2X SSC/0.1% SDS at room temperature, followed by two 15-minute washes with 0.1X SSC/0.1% SDS at 45°C. The filters were exposed to x-ray film overnight. Ausubel et al., supra.

From this initial screening, 52 putative clones were identified. Twenty of these clones were randomly selected, purified by several rounds of re-plating and screening [Maniatis et al., supra] and the insert cDNAs

were subcloned into pBluescript SK(-) by *in vivo* excision [Short *et al.*, *supra*] as recommended by the manufacturer. Plasmid DNA prepared from these clones were analyzed by restriction analysis and/or sequencing. From this survey, a 4.2 kb cDNA representing the largest open reading frame was identified. The cDNA inserts from the other putative clones were shorter, and appeared to be identical based on the nucleotide sequence of the insert ends.

Putative cGS-PDE cDNAs were sequenced by a modification of the Sanger method [Sanger *et al.*, *Proc.Natl.Acad.Sci. USA*, 74:5463-5467] using Sequenase® or Taq Trak® kits as directed by the manufacturer. Templates were prepared from the cDNAs by constructing a series of nested deletions [Henikoff, *Gene*, 28:351-359 (1984)] in the vector, pBluescript SK(-) (Stratagene) using exonuclease III and mung bean nuclease according to the manufacturer's protocol. In cases where overlapping templates were not attained by this method, the cDNAs were cleaved at convenient restriction endonuclease sites and subcloned into pBluescript, or specific oligomers were manufactured to prime the template for sequencing. Single-stranded DNA templates were rescued by isolating the DNA from phagemid secreted by helper phage-infected XL1 cells harboring the pBluescript plasmid [Levinson *et al.*, *supra*] as recommended by the manufacturer (Stratagene). Homology searches of GENBANK (Release 66.0), EMBL (Release 25.0), and NBRF nucleic acid (Release 36.0) and protein (Release 26.0) databases were conducted using Wordsearch, FASTA and TFASTA programs supplied with the Genetics Computer Group software package Devereux *et al.*, *Nuc.Acids Res.*, 12:387-395 (1984).

The nucleotide sequence and deduced amino acid sequence encoded by the large open reading frame of

p3CGS-5 cDNA clone insert is provided in SEQ ID NO: 38 and SEQ ID NO: 39. Starting with the first methionine codon, the cDNA encodes a 921 residue polypeptide with a calculated molecular weight of about 103,000. Although no stop codons precede this sequence, an initiator methionine consensus sequence [Kozak, J.Cell Biol., 108:229-241 (1989)] has been identified. The presence of 36 adenosine residues at the 3′ end of the cDNA preceded by a transcription termination consensus sequence [Birnstiel et al., Cell, 41:349-359 (1985)] suggests that all of the 3′ untranslated sequence of the cGS-PDE mRNA is represented by this clone.

A putative phosphodiesterase-deficient (PPD) strain of S49 cells [Bourne et al., J.Cell.Physiol., 85:611-620 (1975)] was transiently transfected with the cGS-PDE cDNA using the DEAE-dextran method. The cGS-PDE cDNA was ligated into the unique BamHI cloning site in a mammalian expression vector, designated pZEM 228, following a zinc-inducible metallothionine promoter and prior to an SV40 transcription termination sequence. The DNA was purified from large-scale plasmid preparations using Qiagen pack-500 columns as directed by the manufacturer. PPD-S49 cells were cultured in DMEM containing 10% heat-inactivated horse serum, 50 μg/ml penicillin G and 50 μg/ml streptomycin sulfate at 37°C in a water-saturated 7% CO_2 atmosphere. Prior to transfections, confluent 100 mm dishes of cells were replated at one-fifth of the original density and incubated for 24-36 h. In a typical transfection experiment, PPD-S49 cells (50-80% confluent) were washed with Tris-buffered-saline and approximately 2 x 10^7 cells were transfected with 10 μg of DNA mixed with 400 μg of DEAE-dextran in one ml of TBS. The cells were incubated at 37°C for 1 hr with gentle agitation every 20 min. Next, DMSO was added to a final concentration of 10% and

rapidly mixed by pipetting up and down. After 2 min, the cells were diluted with 15 volumes of TBS, collected by centrifugation, and washed, consecutively with TBS and DMEM. The cells were resuspended in complete medium and seeded into fresh 100 mm plates (1-2 x 107 cells/10 ml/plate). After 24 h, the cells were treated with TBS alone, or containing zinc sulfate (final concentration = 125 μM) and incubated for an additional 24 h. The cells were harvested and washed once with TBS. The final cell pellets were resuspended in two mls of homogenization buffer (40 mM Tris-HCl; pH 7.5, 15 mM benzamidine, 15mM β-mercaptoethanol, 0.7 μg/ml pepstatin A, 0.5 μg/ml leupeptin and 5 mM EDTA) and disrupted on ice using a dounce homogenizer. The homogenates were centrifuged at 10,000 x g for 5 min at 4°C and the supernatants were assayed for phosphodiesterase activity and protein concentration.

cGS PDE activity was determined by a previously described method using [^{3}H]cAMP as the substrate as in Martins et al., J.Biol.Chem., 257:1973-1979 (1982). Phosphodiesterase assays were performed in triplicate. The Bradford assay [Bradford, Anal. Biochem., 72:248-254 (1976)] was used to quantitate protein using BSA as the standard.

In the absence of zinc treatment, no increase in basal activity or cGMP-stimulated phosphodiesterase activity was detected in PPD S49 cells transfected with the cGS PDE-ZEM 228 construct or the vector alone. However, zinc-treated cells transfected with cGS-PDE cDNA, but not the vector alone, expressed cGMP-enhanced cAMP phosphodiesterase activity indicating that the cDNA encodes a cGS-PDE. The total activity of the homogenates and 50,000 x g supernatants was not significantly different.

Transient expression of the cGS-PDE cDNA in COS-7 cells was accomplished as in Example I. A 4.2 kb fragment of p3CGS-5 was isolated using HindIII and NotI and was inserted into plasmid pCDM8, which had been digested with the same enzymes. The character of products produced in COS-7 cells transformed with the p3CGS-5/pCDM8 construct is discussed in Example V, infra.

EXAMPLE V

Isolation, Purification, and Partial Sequence Determination of cGS-PDE cDNA from Bovine Brain

A. Isolation of Bovine Brain cGSPDE cDNA Clone, pBBCGSPDE-5

A bovine brain cDNA library constructed with the λ ZAP vector (kindly provided by Ronald E. Diehl, Merck, Sharp & Dohme) was screened with a 450 bp EcoRI/ApaI restriction endonuclease cleavage fragment of the p3CGS-5 cDNA corresponding to (p3CGS-5) nucleotide position numbers 1-452. The probe was prepared using the method of Feinberg et al., supra, and the [^{32}P]-labeled DNA was purified using Elutip D® columns. Plaques (a total of 600,000 plaques on 12-150 mm plates) bound to filter circles were hybridized at 42° overnight in a solution containing 50% formamide, 20 mM Tris HCl (pH 7.5), 1X Denhardt's solution, 10% dextran sulfate, 0.1% SDS and 10^6 cpm/ml [^{32}P]-labeled probe (10^9 cpm/μg). The filters were washed three times for 15 minutes with 2X SSC/0.1% at room temperature, followed by two 15 minute washes with 0.1X SSC/0.1% SDS at 45%. The filters were exposed to x-ray film overnight.

Forty putative clones were picked from this first screen, of which six were randomly selected and purified by several rounds of re-plating and screening

[Maniatis et al., supra]. The insert cDNAs were subcloned into pBluescript SK(-) by in vivo excision as recommended by the manufacturer. Plasmid DNA prepared from cultures of each clone was sequenced from the ends using Sequenase and Taq Trak sequencing kits. The sequence obtained from this experiment confirmed that the bovine brain cDNA clone, pBBCGSPDE-5 was a cGS-PDE cDNA, and that it was different than the adrenal cGS-PDE cDNA at the five-prime end.

Partial sequence analysis of the pBBCGSPDE-5 insert at its 5′ end (encoding the amino terminal region of the protein) revealed the sense strand set out in SEQ ID NO: 40, while sequencing of the 3′ end of the insert revealed the antisense sequence of SEQ ID NO: 41.

B. Isolation of Bovine Brain cGS-PDE cDNA Clone, pBBCGSPDE-7

Each of the forty putative clones selected from the first round of purification described above was spotted individually onto a lawn of host XL1 cells and incubated overnight at 37°. The plaques were screened with a 370 bp PstI/SmaI restriction endonuclease cleavage fragment of the p3CGS-5 cDNA (corresponding p3CGS-5 nucleotide position numbers 2661-3034). The probe was prepared using the method of Feinberg et al., supra, and the [^{32}P]-labeled DNA was purified using Elutip-D® columns. Plaques bound to filter circles were hybridized at 42° overnight in a solution containing 50% formamide, 20 mM Tris-HCl (pH 7.5), 1X Denhardt's solution, 10% dextran sulfate, 0.1% SDS and 10^6 cpm/ml [^{32}P]-labeled probe (10^9 cpm/μg). The filters were washed three times for 15 minutes with 2X SSC/0.1% SDS at room temperature, followed by two 15-minute washes with 0.1X SSC/0.1% SDS at 45°. The filters were exposed to x-ray film overnight.

After several rounds of plating and rescreening, six putative clones were purified and sequenced from the ends. The sequence of the five-prime end of the cDNA clone pBBCGSPDE-7 was identical to clone pBBCGSPDE-5, but not the adrenal gland-derived clone, p3CGS-5. The sequence of the three-prime end of the pBBCGSPDE-7 cDNA clone was identical to the p3CGS-5 insert sequence.

Sequence analysis of the pBBCGSPDE-7 insert revealed the DNA sequence set out in SEQ ID NO: 42 and the amino acid sequence of SEQ. ID NO: 43.

The large open reading frame encodes a 942-residue polypeptide that is nearly identical to the adrenal gland cGS-PDE isozyme (921 residues). The difference in the primary structure of these two isozymes lies in the amino-terminal residues 1-46 of the brain cGS-PDE, and residues 1-25 of the adrenal cGS-PDE. The remaining carboxy-terminal residues of the brain and adrenal cGS-PDE are identical.

For transient expression in COS-7 cells, a 3.8kb fragment of pBBCGSPDE-7 was isolated using HindIII and NotI and inserted into plasmid pCDM8 which had been cut with HindIII and NotI restriction endonucleases. The recombinant pBBCGSPDE-7/CDM8 construct was used to transiently transfect COS-7 cells. The properties of the pBBCGSPDE-7/CDM8 construct and the p3CGS-5/CDM8 construct prepared in Example IV products were subsequently compared. Membrane and supernatant fractions were prepared from extracts of transfected COS-7 cells and assayed for cGS-PDE activity. Both the pBBCGSPDE-7/CDM8 and p3CGS5/CDM8 plasmid constructs produced cGS-PDE activities in COS-7 cell extracts, and most of the activity was detected in the supernatant fractions. However, a 10-fold greater percentage of total cGS-PDE activity was detected in membranes from COS-7 cell

extracts transfected with the pBBCGSPDE-7/CDM8 construct than in membranes prepared from p3CGS-5/CDM8-transfected COS-7 cells. These results indicate that, relative to the adrenal cGS-PDE, the isozyme encoded by the pBBCGSPDE-7 cDNA preferentially associates with cellular membranes.

EXAMPLE VI

Use of cGS-PDE Bovine Adrenal cDNA to Obtain Human cGS-PDE cDNAs

Several human cDNA clones, homologous to a cDNA clone encoding the bovine cyclic GMP-stimulated phosphodiesterase, were isolated by hybridization using a nucleic acid probe derived from the bovine cDNA. A combination of sequence analysis and hybridization studies indicates that these human cDNA clones encompass an open reading frame corresponding to a human phosphodiesterase.

cDNA libraries were probed with DNA from plasmid p3CGS-5 which contains a 4.2-kb cDNA insert encoding the bovine cGS-PDE. This plasmid was digested with the restriction enzymes SmaI and EcoRI. The approximately 3.0 kb fragment derived from the cDNA insert was isolated and purified by agarose gel electrophoresis. This fragment contains the entire open reading-frame of the PDE. The fragment was labeled with radioactive nucleotides by random priming.

The cDNA libraries were plated on a 150 mm petri dishes at a density of approximately 50,000 plaques per plate. Duplicate nitrocellulose filter replicas were prepared. The radioactive nucleic acid probe was used for hybridization to the filters overnight at 42°C in 50% formamide, 5x SSPE (0.9 M NaCl, 0.05 M $NaH_2PO_4 \cdot H_2O$, 0.04 M NaOH, and 0.005 M $Na_2EDTA \cdot 2H_2O$), 0.5% SDS, 100 µg/ml salmon testes DNA, and 5x Denhardt's solution. The filters were

washed initially at room temperature and subsequently at 65°C in 2x SSC containing 0.1% SDS. Positive plaques were purified and their inserts were subcloned into an appropriate sequencing vector for DNA sequence analysis by standard techniques.

First, a λgt10 cDNA library prepared from human hippocampus mRNA (clontech, random and dT primed) was screened. Of the approximately 500,000 plaques examined, 33 hybridized to the probe. One of these phages was digested with EcoRI to remove the cDNA insert. This insert-containing EcoRI fragment was cloned into Bluescript KS that had been digested with EcoRI and then treated with calf intestine alkaline phosphatase. One product of this reaction was the plasmid pGSPDE9.2, which showed two major differences when compared to the bovine cGS-PDE cDNA. The 5'0.4 kb of the pGSPDE9.2 insert diverged from the bovine cDNA. Approximately 0.7kb from the 5' end of the human cDNA there is a 0.7kb region that diverges from the bovine cDNA. This region may be an intron. Twenty-five of the remaining hippocampus plaques that had hybridized to the bovine probe were examined by PCR, hybridization and/or sequencing. None were found to extend through the regions that differed between the bovine and human cDNAs.

Phages λ GSPDE7.1 and λ GSPDE7.4, two other phages from the hippocampus library, were digested with EcoRI and HindIII. Each yielded a 1.8-kb fragment that contains most of the cDNA insert and approximately 0.2-kb of phage lambda DNA. The λ DNA is present in the fragment because in each case one of the EcoRI sites that typically bracket a cDNA insert had been destroyed, possibly when the library was constructed. The EcoRI/HindIII fragments were cloned into Bluescript KS digested with EcoRI and HindIII. This procedure gave rise to the plasmids pGSPDE7.1 and pGSPDE7.4. The cDNA

inserts encode DNA homologous to the 3′ portion of the bovine phosphodiesterase cDNA. Both of the cDNA inserts in these clones begin at an EcoRI site and the sequences are homologous adjacent this site.

Portions of pGSPDE7.1 and pGSPDE7.4 cDNA inserts were sequenced and are identical except for a short region of their 3′ ends. The cDNA insert in pGSPDE7.1 ends with a sequence of approximately 70 adenine bases, while the cDNA insert in pGSPDE7.4 ends with three additional nucleotides not present in pGSPDE7.1 followed by a sequence of approximately 20 adenine bases.

Next, a cDNA library prepared in λ ZapII (Stratagene) from human heart mRNA yielded one hybridizing plaque from the approximately 500,000 screened. The Bluescript SK(-) plasmid pGSPDE6.1 containing the hybridizing insert was excised in vivo from the λ ZapII clone. Sequence analysis showed that the insert is homologous to the bovine phosphodiesterase cDNA. The homologous region spans the position of the EcoRI found in the sequence formed by joining the sequence of the insert from pGSPDE9.2 to the sequence of the insert in pGSPDE7.1 or pGSPDE7.4. Thus, it is thought that the two clones from the hippocampus form a complete open reading frame.

A third λ gt10 library derived from human placenta mRNA yielded five hybridizing plaques from approximately 800,000 screened. These placental cDNA clones were short and their sequences were identical to portions of the hippocampus cDNA pGSPDE9.2. Screening $5x10^5$ plaques from U118 glioblastoma cDNA library, $5x10^5$ from a spleen cDNA library and $5x10^5$ from an adrenal library (Cushings Disease) gave no hybridization plaques.

Given the homology between the bulk of human and bovine cGS-PDE sequence, it was decided to obtain

multiple independent cDNA clones containing the 5′ end of the human cGS-PDE to determine if the 0.4kb 5′ sequence was an artifact. An approximately 0.95kb EcoRI-HindII fragment from the 5′ end of the bovine cGS cDNA plasmid p3cgs5 was random primed and used as a probe to screen a number of human cDNA libraries. Hippocampus library screening was carried out under the same screening conditions as described above. All remaining screenings were carried out as described with respect to human heart cDNA library screenings in Example VII, infra. No positives were obtained screening $5x10^5$ plaques from a human T cell library (Hut78, dT-primed), 10^6 plaques from the hippocampus cDNA library (random and dT-primed), $5x10^5$ plaques from a human liver cDNA library (dT-primed, 5′ stretch, Clontech), $5x10^5$ plaques from a human SW1088 glioblastoma cDNA library (dT-primed), $5x10^5$ plaques from the same heart cDNA library (random and dT-primed), and $1.5x10^6$ plaques from a human lung cDNA library (random primed). Two positives were obtained from screening $5x10^5$ plaques from a human fetal brain cDNA library (random and dT-primed, Stratagene). These were designated as HFB9.1 and HFB9.2.

Bluescript SK(−) plasmids pHFB9.2 and pHFB9.1 were excised *in vivo* from the λZapII clones. DNA sequence analysis revealed that HFB9.1 starts about 80 nucleotides further 3′ than does HFB9.2 and reads into an intron approximately 1.9kb of the way into HFB9.2. HFB9.2 covers the entire open reading frame of the cGS-PDE, but reads into what may be an intron 59 nucleotides after the stop codon. Both of them lack the 5′0.4kb and the presumed intron found in pGSPDE9.2. The entire open reading frame of HFB9.2 was isolated and assembled into yeast expression vector pBNY6N. The resulting plasmid, designated pHcgs6n, includes the coding region of the cDNA as an EcoRI/XhoI insert. DNA and deduced amino acid

sequences for the insert are provided in SEQ.ID No: 44 and 45, respectively.

EXAMPLE VII

Use of CaM-PDE 61 kDa Bovine Brain cDNA to Obtain Human CaM-PDE 61 kDa cDNA

Human cDNA clones, λ CaM H6a and λ CaM H3a, which are homologous to the cDNA encoding the bovine 61 kDa CaM-PDE, were obtained by hybridization using a nucleic acid probe derived from the cDNA encoding the bovine species enzyme. A combination of sequence analysis and hybridization studies indicate that λ Cam H6a contains most of an open reading frame encoding a human CaM-PDE.

The hybridization probe used to isolate the human DNA was derived from first strand cDNA of bovine lung tissue by PCR treatment. More specifically, the 23-mer oligonucleotide designated PCR-2S in Example I (see, SEQ ID NO: 1) was combined in a PCR reaction with bovine lung cDNA and a redundant antisense 23-mer oligonucleotide (PCR-5AS) based on the pCAM insert sequence with

SEQ ID NO: 46

5'TCRTTNGTNGTNCCYTTCATRTT-3'

representing the amino acid sequence

SEQ ID NO: 47

NMKGTTND,

according to the general procedures of Examples I and III, to generate a 1098 bp cDNA fragment representing a large portion of the coding region of the pCAM-40 insert. The PCR products were purified on a 1% agarose gel using

0.4 M Tris-acetate/0.001 M EDTA buffer containing 0.5 µg/ml ethidium bromide. The DNA products were visualized with UV light, cleanly excised from the gel with a razor blade, purified using Geneclean II reagent kit and ligated into EcoRV-cut pBluescript vector DNA.

To determine if the PCR amplification products were CAM-PDE cDNAs, the subcloned PCR DNA products were sequenced from the ends using T3 and T7 promoter primers and either Sequenase or Taq Polymerase sequencing kits. Approximately 250 bases from each end of this DNA were then compared to the amino acid sequence of bovine CAM-PDE, confirming that the PCR DNA product was a partial CAM PDE cDNA. This clone was designated pCAM-1000 and contained a 1.1-kb insert of nucleic acid that corresponds to nucleotides 409 to 1505 of the insert of pCAM-40. pCaM1000 was digested with the restriction enzymes HinDIII and BamHI. The 1.1-kb fragment was purified by agarose gel electrophoresis and then digested with the restriction enzyme AccI. The two fragments were separated and purified by agarose gel electrophoresis. These separated fragments were labeled with radioactive nucleotides by random priming.

Human cDNA libraries were plated on 150 mm petri dishes at a density of approximately 50,000 plaques per dish and duplicate nitrocellulose filter replicas were prepared. Each probe was hybridized to a separate set of the duplicate filters. The filters were hybridized overnight at 65°C in 3x SSC, 0.1% sarkosyl, 50 µg/ml salmon testes DNA, 10x Denhardt's solution, 20 mM sodium phosphate (pH 6.8). They were washed at 65°C in 2x SSC containing 0.1% SDS.

A λ gt10 library prepared from human hippocampus mRNA yielded three hybridizing plaques of the approximately 500,000 screened. Of these three hybridizing plaques, two hybridized to both probes and

the third hybridized to the longer of the two probes. The λ Cam H6a clone contains an approximately 2kb insert that is homologous to the cDNA encoding the bovine clone of pCAM-40.

The λ cam H6a cDNA was subcloned into the plasmid Bluescript KS for sequence analysis. Although the cDNA library had been constructed with EcoRI linkers, one of the EcoRI sites that should have flanked the cDNA insert did not cut with EcoRI. Thus, the cDNA was subcloned as two fragments: an approximately 0.7kb EcoRI/HindIII fragment (pcamH6C) and an approximately 1.6kb HindIII fragment that contained approximately 1.3kb of cDNA and 0.25kb of flanking λgt10 vector DNA (pcamH6B). DNA sequence analysis revealed that it encoded most of a human CaM-PDE homologous to the bovine 61k CaM-PDE, except that the human cDNA appeared to be missing two base pairs in the middle of the coding region. These missing nucleotides correspond to positions 626 and 627 of the human cDNA sequence if it is aligned with the pCAM-40 bovine 61kDa CaM-PDE (SEQ. ID NO: 5 for maximum homology.

Another of the cDNA clones from the hippocampus cDNA library that had been screened with the bovine 61kDa CaM-PDE probes was λcamH2a. It contained an approximately 1.0kb insert. As was the case for λcamH6a cDNA, only one of the two EcoRI sites that should be present at the ends of the insert would cut. The original subcloning and DNA sequence analysis for this cDNA utilized PCR fragments generated with oligos in the flanking λgt10 vector arms. This cDNA overlaps much of the 5′ end of the insert in λcamH6a and contained the additional two nucleotides predicted by the bovine sequence and required to maintain the PDE open reading frame. The λcamH2a insert also appeared to contain two introns; one 5′ of the initiator methionine and one

downstream of the HindIII site. The EcoRI/HindIII fragment from λcamH2a (corresponding to the region covered by pcamH6C) was subcloned into the plasmid Bluescript SK⁻ and designated pcamH2A-16. This was then used as the source of the two additional bp in the construction of yeast expression plasmids described below.

Two different plasmids were constructed for human CaM-PDE expression in yeast. One plasmid, pHcam61-6N-7, contains the entire open reading frame. The second plasmid, pHcam61met140, starts at an internal methionine (beginning at nucleotide position 505) and extends to the end of the open reading frame. These expression plasmids were constructed by modifying the 3′ portion of the open reading frame and then adding the two differently modified 5′ ends to the 3′ end. The sequence of the cDNA insert of pHcam61-6N-7 is set out in SEQ. ID NO: 48 and the deduced amino acid sequence of the CaM-PDE encoded thereby is set out in SEQ. ID NO: 49. During construction of the cDNA insert, the nucleotide at position 826 was altered from T to C, but the encoded amino acid was conserved. Plasmid pHcam61met140, as noted above, has a cDNA insert lacking the first 140 codons of the coding region of the pHcam61-6N-7 but is otherwise identical thereto.

A third cDNA, λcamH3a, contained an approximately 2.7kb insert. This cDNA insert was subcloned for sequence analysis. Although the cDNA library had been constructed with EcoRI, the inserted cDNA in λcamH3a could not be excised with EcoRI. Presumably one of the EcoRI sites was destroyed during the construction of the library. The cDNA insert was excised from the λ clone by digestion with HindIII and EcoRI. This digestion yields two relevant fragments, a 0.6kb HindIII fragment which contains a portion of DNA

from the left arm of λgt10 attached to the cDNA insert and an approximately 2.4kb HindIII/EcoRI fragment containing the remainder of the cDNA insert. These two fragments were assembled in the plasmid Bluescript KS to yield an approximately 3kb fragment. The orientation of the small HindIII fragment was the same as the original λ clone. This subclone is known as pcamH3EF. Although this cDNA hybridizes to the bovine probe from the bovine CaM-PDE 61kDa cDNA, sequence analysis revealed that it appeared to be the product of a different CaM-PDE gene. Plasmid pcamH3EF contains what may be the entire open reading frame and would encode a protein approximately 75% homologous to the protein encoded by the insert of pHcam61-6N-7 over much of its lengths. DNA and deduced amino acid sequences are set out in SEQ. ID NOS: 50 and 51, respectively. The DNA sequence of the region between nucleotide 80 and 100 of pcamH3EF is uncertain. This area is 5′ to the initiator methionine codon and thus does not effect the open reading frame.

An approximately 2.4kb fragment of pcamH3EF was gel purified following digestion with the restriction enzymes HindIII and EcoRI. This fragment was used to screen additional human cDNA libraries in a similar manner to the screen described above. Screening approximately $5x10^5$ plaques from a human heart cDNA library (Stratagene) yielded two plaques that hybridized to the pcamH3EF probe. The Bluescript SK^- plasmid pcamHella was excised in vivo from one of these positive λZapII clones. DNA and deduced amino acid sequences for the cDNA insert are set out in SEQ. ID NO: 52 and 53, respectively. Sequence analysis of pcamHella showed that the insert began at nucleotide position 610 of pcamH3EF and was nearly identical through nucleotide position 2066, at which point the DNA sequence diverged from that of pcamH3EF. The cDNA insert of pcamHella continued for

approximately 0.6kb. The consequence of this divergence is to alter the carboxy terminus of the protein that would be encoded by the open reading frame within the cDNA. The pcamH3EF cDNA could encode a protein of 634 amino acids (MW72,207). Assuming the 5′ end of the pcamHella cDNA is the same as that of the 5′ end of pcamH3EF (5′ to nucleotide position 610), pcamHella could encode a 709 amino acid protein (MW80,759). These divergent 3′ ends may be the consequence of alternative splicing, lack of splicing, or unrelated DNA sequences being juxtaposed during the cloning process.

EXAMPLE VIII

Expression of Bovine and Human PDE cDNAs for Complementation of Yeast Phenotypic Defects

The present example relates to the expression of bovine and PDE clones in yeast demonstrating the capacity of functional PDE expression products to suppress the heat shock phenotype associated with mutation of yeast phosphodiesterase genes and also relates to the biochemical assay of expression products. The host cells used in these procedures were S. cerevisiae yeast strains 10DAB (ATCC accession No. 74049) and YKS45, both of which were pde^{1-} pde^{2-} resulting in a phenotype characterized by heat shock sensitivity, i.e., the inability of cells to survive exposure to elevated temperatures on the order of 55-56°C. In these complementation procedures, the inserted gene product was noted to conspicuously modify the heat shock phenotype. This capacity, in turn, demonstrates the feasibility of systems designed to assay chemical compounds for their ability to modify (and especially the ability to inhibit) the in vivo enzymatic activity of mammalian Ca^{2+}/calmodulin stimulated and cGMP stimulated cyclic nucleotide phosphodiesterases.

A. Yeast Phenotyp Complementation by Expression of a cDNA Encoding CaM-PDE

A 2.2 kb cDNA fragment, adapted for insertion into yeast expression plasmids pADNS (ATCC accession No. 68588) and pADANS (ATCC accession No. 68587) was derived from plasmid pCAM-40 (Example I) by polymerase chain reaction. Briefly, the following PCR amplification was employed to alter the pCAM-40 DNA insert to align it appropriately with the ADH1 promoter in the vectors.

One oligonucleotide primer (Oligo A) used in the PCR reaction

SEQ ID NO: 54

5'-TACGAAGCTTTGATGGGGTCTACTGCTAC-3'

anneals to the pCaM-40 cDNA clone at base pair positions 100-116 and includes a HindIII site before the initial methionine codon. A second oligonucleotide primer (Oligo B)

SEQ ID NO: 55

5'-TACGAAGCTTTGATGGTTGGCTTGGCATATC-3'

was designed to anneal at positions 520-538 and also includes a HindIII site two bases before a methionine codon. The third oligonucleotide

SEQ ID NO: 56

5'-ATTACCCCTCATAAAG-3'

annealed to a position in the plasmid that was 3' of the insert. For one reaction, Oligo A and Oligo C were used as primers with pCAM-40 as the template. The nucleic acid product of this reaction included the entire open reading frame. A second reaction used Oligo B and Oligo

C as primers on the template pCAM-40 and yielded a nucleic acid product that lacked the portion of the cDNA sequence encoding the calmodulin binding domain. These amplified products were digested with HindIII and NotI and ligated to HindIII/NotI-digested yeast expression vectors pADNS and pADANS. Plasmid clones containing inserts were selected and transformed into S. cerevisiae strain 10DAB by lithium acetate transformation.

Transformed yeast were streaked in patches on agar plates containing synthetic medium lacking the amino acid leucine (SC-leucine agar) and grown for 3 days at 30°C. Replicas of this agar plate were made with three types of agar plates: one replica on SC-leucine agar, one replica on room temperature YPD agar, and three replicas on YPD agar plates that had been warmed to 56°C. The three warmed plates were maintained at 56°C for 10, 20, or 30 minutes. These replicas were than allowed to cool to room temperature and then all of the plates were placed at 30°C. Yeast transformed with plasmids constructed to express the CaM-PDE were resistant to the thermal pulse. More specifically, both the construct designed to express the complete open reading frame and that designed to express the truncated protein (including the catalytic region but not the calmodulin binding domain), in either pADNS or pADANS, complemented the heat shock sensitivity phenotype of the 10DAB host cells, i.e., rendered them resistant to the 56°C temperature pulse.

In a like manner, plasmids pHcam61-6N-7 and pHcam61met140 (Example VII) were transformed into yeast host 10DAB. Heat shock phenotypes were suppressed in both transformants.

B. Biochemical Assay of Expression Products

The bovine CaM-PDE expression product was also evaluated by preparing cell-free extracts from the 10DAB yeast cells and measuring the extracts' biochemical phosphodiesterase activity. For this purpose, 200 ml cultures of transformed yeast were grown in liquid SC-leucine to a density of about 6 million cells per ml. The cells were collected by centrifugation and the cell pellets were frozen. Extracts were prepared by thawing the frozen cells on ice, mixing the cells with 1 ml of PBS and an equal volume of glass beads, vortexing them to disrupt the yeast cells, and centrifuging the disrupted cells at approximately 12,000 x g for 5 min to remove insoluble debris. The supernatant was assayed for phosphodiesterase activity.

Extracts of yeast cells, up to 50 µl, were assayed for phosphodiesterase activity in 50mM Tris (pH 8.0), 1.0 mM EGTA, 0.01 mg/mL BSA (bovine serum albumin), [^{3}H]-cyclic nucleotide (4-10,000 cpm/pmol), and 5 mM $MgCl_2$ in a final volume of 250 µl at 30°C in 10 x 75 mm glass test tubes. The incubations were terminated by adding 250 µl of 0.5 M sodium carbonate pH 9.3, 1M NaCl, and 0.1% SDS. The products of the phosphodiesterase reaction were separated from the cyclic nucleotide by chromatography on 8 x 33 mm columns of BioRad Affi-Gel 601 boronic acid gel. The columns were equilibrated with 0.25M sodium bicarbonate (pH 9.3) and 0.5 M NaCl. The reactions were applied to the columns. The assay tubes were rinsed with 0.25M sodium bicarbonate (pH 9.3) and 0.5 M NaCl and this rinse was applied to the columns. The boronate columns were washed twice with 3.75 ml of 0.25 M sodium bicarbonate (pH 9.3) and 0.5 M NaCl followed by 0.5 ml of 50 mM sodium acetate (pH 4.5). The product was eluted with 2.5 ml of 50 mM sodium acetate (pH 4.5) containing 0.1 M sorbitol and collected in

scintillation vials. The eluate was mixed with 4.5 ml Ecolite Scintillation Cocktail and the radioactivity measured by liquid scintillation spectrometry.

Both the construct designed to express the complete bovine open reading frame and that designed to express a truncated protein, in either pADNS or pADANS, expressed active protein as determined by biochemical phosphodiesterase assay of cell extracts. Extracts of 10DAB harboring pcam61met140 yielded measurable phorphodiesterase activity (see, infra, second method of part D) while the extract of 10DAB cells harboring pcamH61-6N-7 lacked detectable activity.

C. Yeast Phenotype Complementation by Expression of a cDNA Encoding a cGS-PDE

The plasmid p3CGS-5, which contains a 4.2-kb DNA fragment encoding the bovine cGS-PDE, was adapted for cloning into pADNS and pADANS by replacing the first 147 bases of the cDNA with a restriction site suitable for use in insertion into plasmids. The oligonucleotide BS1, having the sequence

SEQ ID NO: 57

5'TACGAAGCTTTGATGCGCCGACAGCCTGC,

encodes a HindIII site and anneals to positions 148-165 of the cDNA insert. An oligonucleotide designated BS3

SEQ ID NO: 58

GGTCTCCTGTTGCAGATATTG,

anneals to positions 835-855 just 3' of a unique NsiI site. The resulting PCR-generated fragment following digestion with HindIII and NsiI was then ligated to HindIII- and NsiI-digested p3CGS-5 thereby replacing the

original 5′ end of the bovine cDNA. A plasmid derived from this ligation was digested with HindIII and NotI to release the modified cDNA insert. The insert was cloned into pADNS and pADANS at their HindIII and NotI sites. These plasmids were then transformed into the yeast strain 10DAB by the lithium acetate method and the transformed cells were grown and subjected to elevated temperatures as in Section A, above. Yeast transformed with plasmids constructed to express the bovine cGS-PDE were resistant to the thermal pulse.

In a like manner, plasmid pHcgs6n (Example VI) was transformed into yeast host strain YKS45 by lithium acetate transformation. Heat shock analysis was performed as above except that the plates were initially grown two days at 30°C and the warmed plates were maintained at 56°C for 10, 20, 30 and 45 minutes. Yeast transformed with the plasmid designed to express the full length human cGS-PDE was resistant to thermal pulse.

D. Biochemical Assay of Expression Product

The expression of the bovine cGS-PDE was also evaluated by preparing cell-free extracts from the yeast and measuring the extracts' biochemical phosphodiesterase activity. For this purpose, 50 ml cultures of transformed 10DAB yeast cells were grown in liquid SC-leucine to a density of about 10 million cells per ml. Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1986). The cells were collected by centrifugation, the cell pellets were washed once with water, and the final cell pellets were frozen. To prepare an extract, the frozen cells were thawed on ice, mixed with 1 ml of PBS and an equal volume of glass beads, vortexed to disrupt the yeast cells, and centrifuged to remove debris. The supernatant was then assayed for phosphodiesterase

activity as in Section B, above. Constructs in either pADNS or pADANS expressed active protein as determined by biochemical phosphodiesterase assay of cell extracts.

YKS45 transformed with plasmid pHcgs6n were grown in SC-leu medium to 1-2x10^7 cells/ml. The cells were harvested by centrifugation and the cell pellets were frozen. A frozen cell pellet, typically containing 10^{10} cells, was mixed with lysis buffer (25mM Tris HCl pH 8, 5mM EDTA, 5mM EGTA, 1mM o-phenathroline, 0.5mM AEBSF, 0.01mg/mL pepstatin, 0.01mg/mL leupeptin, 0.01mg/mL aprotinin, 0.1% 2-mercaptoethanol) to bring the total volume to 2.5 ml. The mixture was thawed on ice and then added to an equal volume of glass beads. The cells were disrupted by cycles of vortexing and chilling on ice, then additional lysis buffer was mixed with the disrupted cells to bring the total lysis buffer added to 5 ml. The suspension was centrifuged for 5 min. at 12,000xg. The supernatant was removed and either assayed immediately or frozen rapidly in a dry ice ethanol bath and stored at -70°C.

Phosphodiesterase activity was assayed by mixing an aliquot of cell extract in (40mM Tris-Cl pH 8.0, 1.mM EGTA, 0.1mg/mL BSA) containing 5mM $MgCl_2$ and radioactive substrate, incubating at 30°C for up to 30 min. and terminating the reaction with stop buffer (0.1M ethanolamine pH 9.0, 0.5M ammonium sulfate, 10mM EDTA, 0.05% SDS final concentration). The product was separated from the cyclic nucleotide substrate by chromatography on BioRad Affi-Gel 601. The sample was applied to a column containing approximately 0.25 ml of Affi-Gel 601 equilibrated in column buffer (0.1M ethanolamine pH 9.0 containing 0.5M ammonium sulfate). The column was washed five times with 0.5ml of column buffer. The product was eluted with four 0.5 ml aliquots of 0.25M acetic acid and mixed with 5 ml Ecolume (ICN

Biochemicals). The radioactive product was measured by scintillation counting. Extracts from yeast expressing the human cGS-PDE hydrolyzed both cyclic AMP and cyclic GMP, as expected for this isozyme.

EXAMPLE IX

Tissue Expression Studies Involving CaM-PDE and cGS-PDE Polynucleotides

A. Northern Blot Analysis

DNAs isolated in Examples I, III, and IV above were employed to develop probes for screening total or poly A-selected RNAs isolated from a variety of tissues and the results are summarized below.

1. Northern analysis was performed on mRNA prepared from a variety of bovine adrenal cortex, adrenal medulla, heart, aorta, cerebral cortex, basal ganglia, hippocampus, cerebellum, medulla/spinal cord, liver, kidney cortex, kidney medulla, kidney papillae, trachea, lung, spleen and T-lymphocyte tissues using an approximately 3kb radiolabeled cDNA fragment isolated from plasmid p3CGS-5 upon digestion with EcoRI and SmaI. A single 4.5kb mRNA species was detected in most tissues. The size of the cGS-PDE mRNA appeared to be slightly larger (approximately 4.6kb) in RNA isolated from cerebral cortex, basal ganglia and hippocampus. The cGS PDE mRNA was most abundant in adrenal cortex. It was also abundant in adrenal medulla and heart. It appeared to be differentially expressed in anatomically distinct regions of the brain and kidney. Among RNAs isolated from five different brain regions, cGS PDE mRNA was most abundant in hippocampus, cerebral cortex, and basal ganglia. Very little cGS PDE transcript was detected in cerebellum or medulla and spinal cord RNAs. Although the cGS PDE mRNA was detected in all regions of the kidney, it appeared to be most abundant in the outer red medulla

and papillae. The cGS PDE mRNA was also detected in liver, trachea, lung, spleen, and T-lymphocyte RNA. Very little cGS PDE mRNA was detected in RNA isolated from aorta.

2. Radiolabeled DNA probes were prepared from random hexamer primed fragments extended on heat denatured 1.6kb EcoRI restriction endonuclease fragments of the cDNA insert of plasmid pCAM-40. In Northern analysis, the DNA probes hybridized with 3.8 and 4.4kb mRNAs in brain and most of the other tissues analyzed including cerebral cortex, basal ganglia, hippocampus, cerebellum, medulla and spinal cord, heart, aorta, kidney medulla, kidney papillae, and lung. Hybridization of probe with the 3.8kb mRNA from liver, kidney cortex and trachea was only detected after longer autoradiographic exposure.

3. Northern blot analysis of mRNA from several tissues of the central nervous system was carried out using a subcloned, labeled p12.3a DNA fragment (containing most of the conserved PDE catalytic domain) as a probe. The most intense hybridization signal was seen in mRNA from the basal ganglia and strong signals were also seen in mRNA from other tissues including kidney papilla and adrenal medulla.

B. RNAse Protection

1. Three antisense riboprobes were constructed. Probe III corresponds to the catalytic domain-encoding region of p3cGS-5 (273 bp corresponding to bases 2393 through 2666 of SEQ. ID NO: 38); probe II to the cGMP-binding domain encoding (468 bp corresponding to bases 959 through 1426; and probe I to the 5′ end and portions of amino terminal-encoding region (457 bases corresponding to bases 1 through 457).

Total RNAs extracted from all of the examined tissues completely protected probes II and III. Nearly complete protection (457 bases) of riboprobe I with RNAs isolated from adrenal cortex, adrenal medulla, and liver was also observed. However, RNA isolated from cerebral cortex, basal ganglia, and hippocampus only protected an approximately 268-base fragment of riboprobe I. A relatively small amount of partially protected probe I identical in size with the major fragments observed in the brain RNA samples was also detected in RNAs isolated from all of the examined tissues except liver. Interestingly, heart RNA yielded both completely protected (457 base) riboprobe and, like brain RNA, a 268-base fragment. Unlike the protection pattern observed using RNAs isolated from any of the other tissues, however, the partially protected riboprobe I fragment appeared to be more abundant. The results suggest that two different cGS-PDE RNA species are expressed.

2. Radiolabeled antisense riboprobes corresponding to a portion of either the CaM-binding domain on the catalytic domain of CaM-PDE were constructed from restriction endonuclease cleavage fragments (AccI/SstI and Tth111I/HincII) of pCAM-40cDNA. Total RNAs isolated from five different brain regions (cerebral cortex, basal ganglia, hippocampus, cerebellum, and medulla/spinal cord) completely protected the antisense riboprobes encoding both the CaM-binding and catalytic domains. Total RNAs from heart, aorta, lung, trachea and kidney completely protected the riboprobe corresponding to the catalytic domain but only protected about 150 bases of the CaM-binding domain riboprobe, suggesting that an isoform structurally related to the 61kD CaM-PDE is expressed in these tissues.

3. Antisense riboprobes were generated based on plasmid p12.3a and corresponding to bases -1 through 363 and 883-1278 of SEQ. ID NO: 26. The former probe included 113 bases of the 5' noncoding sequence as well as the start methionine codon through the putative CaM-binding domain, while the latter encoded the catalytic domain. Among all tissues assayed, RNA from basal ganglia most strongly protected each probe. Strong signals of a size corresponding to the probe representing the amino terminus were observed in protection by cerebral cortex, cerebellum, basal ganglia, hippocampus and adrenal medulla RNA. No protection was afforded to this probe by kidney papilla or testis RNA even though the tissue showed signals on the Northern analysis and RNAse protection of the conserved domain probe, suggesting that a structurally related isozyme is expressed in this tissue.

While the present invention has been described in terms of specific methods and compositions, it is understood that variations and modifications will occur to those skilled in the art upon consideration of the invention. Consequently only such limitations as appear in the appended claims should be placed thereon. Accordingly, it is intended in the appended claims to cover all such equivalent variations which come within the scope of the invention as claimed.